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Diana Kantarovich April 10, 2017

The Effects of Continuous Positive Airway Pressure (CPAP) Treatment on Fear Learning in Veterans with Obstructive Sleep Apnea with and without Co-morbid Posttraumatic Stress Disorder (PTSD)

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Abstract

The Effects of Continuous Positive Airway Pressure (CPAP) Treatment on Fear Learning in Veterans with Obstructive Sleep Apnea with and without Co-morbid Posttraumatic Stress Disorder (PTSD)

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Posttraumatic stress disorder (PTSD) is a debilitating neurological condition that results from exposure to an extremely stressful and dangerous event. Primarily, this disorder is characterized by three major symptom clusters: re-experiencing, avoidance, and physiological alterations. Intrusive nightmares as well as co-morbid breathing impairments during sleep, such as obstructive sleep apnea (OSA), lead to sleep disruptions. Sleep has been widely associated with memory consolidation and learning. Considering patients diagnosed with PTSD have an impaired ability to discriminate between danger and safety cues, we propose that these impairments might be enhanced by reductions in sleep, more specifically, by disruptions in rapid eye movement (REM) sleep. Our study identified 41 veterans from the Sleep Clinic (Veterans Affairs Long Beach Healthcare System, Long Beach, CA). Twenty-three were diagnosed with OSA and were treated using continuous positive airway pressure (CPAP) for approximately eight weeks. We used a protocol where conditioned fear was acquired throughout the AX+/BX- Discrimination paradigm. During Acquisition, two shapes were presented together and paired with an aversive airblast to the larynx (AX+) and another set of shapes were presented and not reinforced by the unconditioned stimulus (BX-). Stimuli A and B were presented together to measure the inhibition of conditioned fear. Additionally, participants underwent Extinction Learning, where the conditioned stimulus was no longer reinforced, and after 24 hours they underwent the Recall of Extinction Test. These assessments

were conducted both pre- and post-CPAP treatment. All patients showed a robust fear-potentiated startle to the reinforced stimulus. Our results demonstrated that CPAP treatment enhanced the ability to inhibit fear responses in patients with OSA. Additionally, participants with OSA showed rapid extinction of the previously conditioned stimulus both prior and after CPAP treatment. However, patients with OSA and co-morbid PTSD had an impaired ability to extinguish prior to the CPAP treatment. Our study advocates for further research in this group of veterans in order to better understand the underlying neurobiology leading to the results found in our study and to improve current protocols for treatment in patients with these disorders.

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Introduction

Posttraumatic stress disorder (PTSD) is a heterogeneous disorder that is usually caused by an event that evokes feelings of fear and helplessness in the affected individuals. PTSD affects 8% of the general population and occurs at a much higher percentage in individuals who experienced trauma (Kessler et al., 1995). PTSD is characterized by intrusive reminders of the trauma, avoidance of trauma-related experiences, as well as hyperarousal. The heterogeneity of this condition is demonstrated through the involvement of multiple neurobiological mechanisms, which are believed to underlie the development of PTSD. This condition is often observed in active duty and military Veterans and in civilian individuals living in low socio-economical urban environments (Kean et al., 2009). Individuals with PTSD are often unable to effectively discriminate between safe and dangerous cues, i.e., even when exposed to situations in a safe environment, a salient cue might trigger memories of the previous traumatic experience (Jovanovic et al., 2012). Due to the association made between trauma-related cues and innocuous safe cues, patients with PTSD commonly have impaired extinction learning and memory as well as poor sleep quality (Milad et al., 2008; Milad et al., 2009). Multiple studies have explored methods to better understand the underlying neurobiological mechanisms leading to the development of PTSD (Pitman et al., 2012; Briscione et al., 2014).

Sleep disruptions and nightmares that involve repetition of the traumatic event are important symptoms for PTSD according to the Diagnostic and Statistical Manual for Mental Disorders, Version 5 (DSM-5; American Psychiatric Association, 2013). Hyperarousal and impaired breathing during sleep (i.e., apnea) appear to exacerbate the symptoms of PTSD, in

part, due to the chronic insomnia that can ensue (Krakow et al., 2000, Krakow et al., 2001). Furthermore, Mellman and colleagues (2002) have reported that the development of PTSD symptoms in trauma-exposed patients was associated with a diminished and more fragmented REM sleep. Sleep fosters the consolidation of memory because this represents an extended period of time during which memories are more resistant to intrusions from competing or disrupting events and thus can become more stable (Walker and Stickgold 2005). Poor sleep quality has the potential to alter an individual's ability to learn new associations. For example, the ability to differentiate between dangerous and safety cues may become impaired (Walker and Stickgold, 2005). Therefore, we hypothesized that improving sleep quality in patients with impaired breathing during sleep and co-morbid PTSD would also lead to improvements in their fear learning.

There have been contradictory research results related to the importance of REM sleep and slow wave sleep (SWS) in humans. However, it appears that the impairments to these two types of sleep differently affect subtypes of memory being consolidated. For example, Plihal and Born (1999) reported that early sleep, which is characterized by SWS, improved performance in declarative tasks (e.g., recall of paired-associated list tasks). However, late sleep (characterized by increase in REM sleep) significantly improved memory for procedural tasks (e.g., mirror tracing skills). Furthermore, declarative memory processing is different depending on whether the memory is of a neutral content or an emotionally arousing event (Cahill and McGaugh 1998). The amygdala, a primary limbic brain region, is actively involved in modulating emotionally arousing, declarative memory processing which also recruits the hippocampus, striatum, and neocortex (Cahill and McGaugh 1998). Studies demonstrated that REM sleep, in

particular, plays an important role for consolidating emotional memories, including those involving learned fear (Wagner et al., 2001; Goldstein and Walker 2014).

When investigating the association between REM sleep and memory learning or consolidation, investigators have examined three primary paradigms: spatial, cued, or contextual learning (Silvestri 2005). Spatial learning involves the processing of a stimulus and its orientation within a specific environment. Cued learning occurs when a stimulus, such as a tone, is repeatedly paired with an aversive event (e.g., footshock in rodent paradigms) during conditioning. This type of learning involves the limbic system of the mammalian brain including the amygdala (Phillips and LeDoux, 1992). Finally, contextual learning relies on the subject's ability to associate a specific environment with an aversive event and not only involves the amygdala but also the hippocampus (a primary brain region implicated in the processing of memory; Silvestri 2005, Phillips and LeDoux 1992). In relation to the current thesis study, sleep deprivation in rats prior to fear conditioning impaired hippocampus-mediated contextual learning but did not affect amygdala-driven cued fear learning (Ruskin et al., 2004). Hence, we expected our participants to successfully learn the fear-conditioning paradigm, which would be demonstrated through their fear-potentiated startle responses.

Nevertheless, several pre-clinical, translational animal studies have suggested that memory extinction becomes impaired after sleep deprivation, specifically REM sleep deprivation (Silvestri 2005, Fu et al., 2007). Silvestri (2005) demonstrated that extinction of a cued task was impaired by a 6-hour reduction of REM sleep in rats. Additionally, a study by Fu and others (2007) showed that a decrease in REM sleep after cued fear conditioning impaired fear extinction learning in rats. This was demonstrated by the significant increase in freezing

that was seen after REM sleep deprivation during the Recall of Extinction Test (Fu et al., 2007). Finally, a study in healthy human participants demonstrated that REM sleep promotes good discrimination between dangerous and safety stimuli (Menz et al., 2016). Adequate duration of REM sleep reduced the return of fear after extinction of the danger stimuli, suggesting the participants consolidated the extinction task better during REM sleep (Menz et al., 2016).

An emerging body of research suggests that there is a clinically significant association between PTSD and sleep-disordered breathing, such as obstructive sleep apnea (Krakow et al., 2000; Lettieri et al., 2016). Obstructive sleep apnea (OSA) is a condition where an individual experiences intermittent, cyclical cessation or reduction of airflow, which results from an obstruction of the extra-thoracic upper airway (Dempsey et al., 2010). The decrease in ventilation leads to brief arousal from sleep and sleep state fragmentation throughout the night (Dempsey et al., 2010). Symptoms associated with OSA can include snoring and daytime sleepiness (Krakow 2006). Ultimately, OSA can lead to other health complications such as cardiovascular abnormalities (Dempsey et al., 2010). Sleep disordered breathing is also associated with poor physical and mental health, hence it is important to treat upon diagnosis (Krakow et al., 2002, Sharafkhaneh et al., 2005). While sleep-disordered breathing occurs both during REM sleep and non-REM sleep, OSA appears to be more severe during REM sleep (Peregrim et al., 2013). Furthermore, Koo and Nam (2016) demonstrated that patients with little REM sleep had a greater apnea-hypopnea index. Continuous positive airway pressure (CPAP) is commonly used as treatment for OSA. However, patients with OSA and co-morbid PTSD have decreased adherence to the CPAP treatment and worse responses to the treatment, thus illustrating the difficulty in treating OSA in patients with co-morbid PTSD (Lettieri et al.,

2016). Nevertheless, it is important to implement breathing treatments in this population as improvements in sleep quality may lead to decreases in PTSD symptom severity and improvements in life quality (Krakow et al., 2000; Lettieri et al., 2016).

To better understand the psychophysiological responses associated with PTSD, many researchers have used Pavlovian fear conditioning which typically employs the pairing of specific cues with aversive outcomes as well as a different set of cues that have no outcomes and are, by nature, safety cues. The amygdala plays a crucial role in mediating an animal's ability to display conditioned fear responses (e.g., freezing, enhanced startle). More specifically, responses during fear conditioning can be attributed to the central nucleus of the amygdala, which projects to the hypothalamic and brainstem regions (Davis et al., 1997). An often-employed psychophysiological paradigm for studying fear processing in rodents and humans is known as fear-potentiated startle. By definition, fear-potentiated startle is the increase in the magnitude of frequency of the acoustic startle reflex when elicited in the presence of a conditioned stimulus (CS), which is paired with an aversive stimulus (unconditioned stimulus, US; Davis et al., 1993). The startle reflex, which is present in all mammals, can be measured in humans by recording the electromyogram (EMG) responses of *orbicularis oculi* muscle contractions (eye blink). This response is elicited by an acoustic stimulus when participants are anticipating the onset of an aversive stimulus (Morgan et al., 1995; Davis et al., 1993). Interestingly, patients with PTSD show an increased startle response both at baseline (Morgan et al., 1995) and when exposed to stimuli that have been reinforced with a US (Norrholm et al., 2011; Glover et al., 2011).

In our set of experiments, we will be using a conditional discrimination paradigm

(termed AX+/BX-) that has been used to better understand biological mechanisms underlying PTSD and was originally translated into human research from rat models and non-human primates (Jovanovic et al., 2005, Myers and Davis 2004, Kamaza et al., 2013). This method provides a psychophysiological evaluation of fear potentiation and fear inhibition in humans (Jovanovic et al., 2005). The acoustic startle response is a reflexive contraction of the skeletal musculature that occurs after a sudden auditory stimulus. Startle studies in PTSD have shown great effects with eye blink electromyography (EMG) and skin conductance responses to startle stimuli (Jovanovic et al., 2005; Norrholm et al., 2011; Briscione et al., 2014; Norrholm et al., 2015). During Acquisition phase one set of shapes (CS), cue AX, is paired with the delivery of an aversive stimulus (air blast to the larynx, US, AX+) and a different set of shapes, cue BX, is shown without the stimulus (BX-). X is a common stimulus to both sets of shapes to prevent any effects of configural learning (i.e., one shape is dangerous but two shapes are safe). Fear inhibition is then measured in the transfer test phase by presenting the danger signal (A) together with the safety signal (B) (Jovanovic et al., 2005). Previous research has shown that patients with PTSD have an impaired ability to transfer a learned safety cue compared to healthy controls and therefore have poor fear inhibition (Jovanovic et al., 2012).

Extinction of fear is a critical learning process in which an organism learns that a stimulus that was once dangerous is now safe. It is also the foundation that underlies clinical exposure treatments that are used as a basis for PTSD treatment to suppress learned fear responses. In this behavioral paradigm a dangerous stimulus (a light or geometric shape), which had previously been paired with an aversive stimulus (a shock or airblast), is non-reinforced repeatedly. Studies suggest that those with PTSD are less able to extinguish or maintain

extinction learning of acquired fear responses (Milad et al., 2008; Norrholm et al., 2011; Pitman et al., 2012). A study of traumatized civilians in a large urban environment by Norrholm and colleagues (2011) showed that individuals with significant PTSD symptoms exhibit higher fear-potentiated startle responses to the previously reinforced danger signals during extinction as compared to those without PTSD symptoms (Norrholm et al., 2011). This inability to inhibit and extinguish conditioned fear plays a role in the patients' symptoms such as re-experiencing, which can involve nightmares leading to poor sleep.

The proposed study will assess both physiological and clinical data to evaluate the effects of CPAP treatment in patients with OSA with and without PTSD. Treatment of OSA with CPAP has been previously associated with sleep improvements, which could additionally alleviate symptoms of other illnesses, such as PTSD, in a traumatized population (Mysliwiec and Roth 2013). Furthermore, a resent study by El-Solh and colleagues (2017) showed that CPAP treatment in veterans with OSA and PTSD led to improvements in clinically assessed PTSD symptoms (PTSD checklist questionnaire, PCL). We will investigate changes in startle by measuring EMG responses in participants during the AX+/BX- conditional discrimination paradigm, a test of safety learning, and extinction learning. We hypothesize that patients diagnosed with both OSA and PTSD will have impaired fear extinction prior to CPAP treatment. However, after CPAP treatment we expect the startle measures to decrease, as a result of improved sleep quality and better memory consolidation. Additionally, in accordance with previous studies, we expect to see an improvement in their PTSD symptom severity. Finally, we expect that patients diagnosed with OSA without PTSD will display a significant decreased fear-potentiated startle during the extinction phase after CPAP treatment.

Methods

Participants

Participants were recruited from the Sleep Clinic (Veterans Affairs Long Beach Healthcare System, Long Beach, CA) prior to initiation of CPAP treatment for OSA. Forty-one participants with OSA participated in the study after signing a consent form. The sample included 37 male and 4 female participants with ages ranging from 24-56 years. Patients were diagnosed with PTSD by clinicians using the Clinician-Administered PTSD Scale for DSM-5 (CAPS-5). All subjects were screened for visual and auditory impairment. Participants were excluded if they reported alcohol or substance abuse within the past 3 months or were diagnosed with a medical or psychiatric condition that could impair sleep (including the diagnosis of Major Depression). Also exclusionary for study participation was the use of medications that fell under any of the following classifications, or had the potential to affect psychophysiological measures and learning: (i) Benzodiazapene use of greater than 10 mg/day (or other diazepam equivalents), (ii) Opiates, (iii) Antipsychotics, or (iv) Anticholinergics.

Clinical Assessment and Sleep Study Measures

Self-report questionnaires were administered both pre- and post-CPAP treatment to examine correlations between sleep quality, history of trauma exposure, and depressive symptomatology. The questionnaires administered were: BDI-II (Beck Depression Inventory-II), Pittsburgh Sleep Quality Index (PSQI), and PCL-C (PTSD Checklist-Civilian version). CPAP adherence was electronically captured by a computer chip embedded in the CPAP device

throughout the study. Additionally, we were able to assess the lowest levels of oxygen saturation, apnea-hypopnea indices, and sleep efficiency.

Startle Procedure

According to methods established by Grillon and Ameli (1998) and previous studies in our lab to gather physiological startle responses during Acquisition, Extinction Learning and Recall of Extinction experiments, the unconditioned stimulus (US) used in this paradigm was a 250 ms airblast with an intensity of 140 p.s.i. directed to the larynx (Norrholm et al., 2006). Airblasts were emitted by a compressed air tank connected to polyethylene tubing and controlled by a solenoid switch. Conditioned stimuli (CS) used were colored shapes, which appeared on a computer screen (both the colors and shapes were counterbalanced across subjects). Stimuli were presented using SuperLab 4.0 for Windows (Cedrus, Inc.) and synchronized with psychophysiological data acquisition using a DIO card (Measurements Computing, Inc.). Acoustic startle probes (108 dB white noise blasts) were delivered throughout the experiments with nearly instantaneous rise and fall times lasting 100 ms; these were delivered binaurally through headphones.

Day 1

Based on methods used in previous studies by Jovanovic and colleagues (2005, 2009), participants underwent AX+/BX- conditioning. Figures 1 and 2 illustrate schematic representations of the AX+/BX- Discrimination Test paradigm. The test session was initiated with a habituation phase, which consisted of six acoustic startle probes presented alone, known as noise alone (NA) trials, to reduce initial startle reactivity. The pre-exposure phase, was

characterized by the presentation of the different shapes (A, B, and X) without pairing with the aversive stimulus (US). The Fear Acquisition (or Conditioning) Phase included 3 blocks with 12 trials (4 AX+, 4 BX-, and 4 NA trials) in each block for a total of 48 trials (including pre-exposure phase). Each compound CS had one novel cue (A or B) and one common cue "X." In the AX+ trials, two shapes of different colors (stimuli A and X) were presented together with a "+" between them in order for the shapes to be processed together configurally. These shapes were presented for 6 seconds. The unconditioned stimulus co-terminated with the presentation of the shapes 500 ms after the presentation of the auditory startle probe. During the 4 BX- trials stimuli B and X (different colored shapes) were not paired with the US. Intertrial intervals were randomized between 9 and 22 seconds. The fear inhibition testing phase, also known as the Transfer of Inhibition Test, occurred immediately after the conditioning phase and consisted of a block of 3 NA trials and 3 trials with A and B stimuli presented together (AB). The AB trials also contained two different colored shapes, however these were not reinforced with the aversive stimulus (US).

After a ten-minute rest period following the completion of the AX+/BX- phase, or fear Acquisition session, the same shapes (stimuli A, B and X) were presented in random order during the extinction phase. The Extinction phase included 6 blocks of 12 trials (4 AX, 4 BX, and 4 NA trials). During extinction, both the AX and BX trials were not reinforced with the US. Startle probes were still delivered on each trial for 40 ms, and occured 5460 ms after shape presentation was initiated. Intertrial intervals were again randomized between 9 and 22 seconds.

Day 2

The Extinction Recall (termed Recall) session occurred 24 hours after the Extinction Learning phase. During the Recall session, the same shape stimuli (A, B and X) were presented using the same design as the extinction trial; however, only 1 block was included. The Recall session was administered to assess the participants' ability to retain the extinction memory formed during the Extinction Learning phase.

Follow-up

Following the pre-treatment assessment, participants underwent continuous positive airway pressure treatment to correct sleep disturbances caused by obstructive sleep apnea. Post-treatment assessments occurred only after the CPAP treatment had resulted in effective restoration of sleep, as measured internally by the CPAP device. The duration was based on the patients' responses to the CPAP treatment with the average length of treatment at approximately eight weeks (between 4-12 weeks). In the post-treatment assessment sessions, the colored shapes (conditioned stimuli) used were different than those used during the pre-treatment psychophysiological assessments. Acquisition and Extinction sessions were conducted on the first day and Recall session was conducted on the second day, similarly to the initial assessment methods.

Physiological Measurements

Acoustic startle responses were measured through participants' electromyographic (EMG) activity (eye blink responses) elicited by the sudden tone probes during the

psychophysiological assessment. EMG startle eye blink responses were recorded using two electrodes placed over the *orbicularis oculi* muscle of the left eye. Two 5 mm Ag/AgCl electrodes filled with electrolyte gel were positioned approximately 1 cm under the pupil and 1 cm below the lateral canthus and a ground electrode was placed behind the right ear over the mastoid. Impedance between the two electrodes was measured and deemed acceptable if below 10 kilo-ohms. EMG activity was amplified and digitized using a computerized EMG startle response monitoring system (SR-LAB, San Diego Instruments). The EMG signal was filtered with 30 and 1000 Hz.

Statistical analysis

Raw startle magnitude was amplified and digitized by the Biopac AcqKnowledge recording software suite (BIOPAC, Goleta, CA). The delivery of the acoustic startle probe and its associated digital marker was synchronized. Data were exported to Mindware (MindWare, Gahanna, OH) software for data reduction and generation of analyzable variables. Blink responses were evaluated based on previous work from our group (Jovanovic et al., 2009). During this analysis one participant was removed because his data was considered to be an outlier (greater than two standard deviations from the mean). Fear-potentiated startle to the reinforced stimulus (AX+) and discrimination between the danger (AX) and safety (BX) cues was determined using repeated measures analysis of variance (RM-ANOVA). The Trial Type consisted of two levels (NA and AX to measure fear-potentiated startle; AX and BX to measure discrimination between cues). The Group consisted of 3 levels (block 1, block 2 and block 3) and accounted for changes in startle responses to stimuli between each block throughout

Acquisition phase. The data was split into the pre- and post-CPAP treatment groups, describing the timepoints for the participant's assessments. The dependent variable (fear-potentiated startle) was calculated using the Difference Score [mean startle magnitude in the presence of the conditioned stimulus (shape presentation) within each block of the Acquisition phase]-[the mean of the startle magnitude to noise probe alone, NA] as in Norrholm et al. (2011).

Examining the dependent variables across the three blocks allowed us to visualize learning effects during Acquisition phase both in terms of the development of fear-potentiated startle (NA vs AX) and the discrimination between danger (AX) and safety (BX; Norrholm et al., 2011). For the transfer of inhibition test, we compared the mean magnitude of fear-potentiated startle in response to AB stimulus to the mean AX potentiated startle responses during the third block of Fear Acquisition phase. For the Extinction Learning Test we measured the startle Difference Scores at each block of the test and used ANOVA to look at the effect by Block. Recall of Extinction (24 hours after Extinction) was measured by comparing the effect by Block between the last block of Extinction (EXT 6) and the magnitude of fear-potentiated startle during the Recall session.

The data analyses were conducted using SPSS 10.0 for Macintosh (SPSS, Inc). Graphs were made using Prism 5 for Mac OSX.

Results

Demographics and Clinical Assessment

Initially, the Sleep Clinic at the Long Beach VA completed a retrospective chart review of Veteran patients that were diagnosed with OSA and demonstrated significant PTSD symptoms. The clinic investigated the responses from the PCL and BDI questionnaires in this group of patients before and after administering CPAP treatment. Results from this exploratory study revealed that CPAP could be beneficial in reducing PTSD and depression symptoms (Table 1). Reduction in PTSD severity as measured by the PCL scores was 43%. These results lead to the development of our study to further explore the mechanisms involved in such improvements.

In the current study, of the 41 participants, 23 had OSA and were treated with CPAP treatment, 9 had OSA and were not treated, and 8 were deemed psychiatrically healthy controls (1 patient excluded). When investigating all of the subjects in our study, only 8 participants were diagnosed with OSA and co-morbid PTSD, and 7 of these patients were given CPAP treatment. Compliance was recorded using the CPAP device and some subjects were shifted to the untreated group due to their lack of compliance. Of those subjects treated, one participant was considered an outlier and was also excluded upon data analysis. The average PCL scores reported by participants prior to CPAP treatment were 35, while post-treatment the average results were 32. Table 2 illustrates the demographic and clinical information of the participants in our study.

Acquisition Phase: Fear-Potentiated Startle

Prior to CPAP treatment, participants showed a robust fear-potentiated startle to the reinforced conditioned stimulus (AX) as compared to noise alone (NA, or baseline), across the three blocks of the Acquisition session with main effects of Block ($F(1,17) = 4.79$, $p = 0.04$) and Trial Type ($F(1,17)=17.2$, $p = 0.001$). Similarly, at the post-treatment timepoint, participants also showed significant fear-potentiation to AX across the three blocks of the Acquisition session with main effects of Block ($F(1,12) = 5.21$, $p = 0.04$) and Trial Type ($F(1,12) = 6.0$, $p = 0.03$). Fear-potentiated startle responses are summarized for the pre- and post-CPAP treatment conditions across the Acquisition phase in Figure 3.

Acquisition Phase: AX+/BX- Discrimination

We next measured participant fear-potentiated startle responses to the reinforced CS+ and non-reinforced CS- during an Acquisition phase before and after CPAP treatment. Prior to treatment, participants demonstrated significant discrimination between the CS+ and CS- as evidenced by a main effect of Trial Type across the last two blocks of the Acquisition phase ($F(1,17) = 10.0$, $p = 0.006$); a point during learning at which we would expect discrimination to be at its peak (see Norrholm et al., 2015). Following treatment, we also observed significant discrimination between the CS+ and CS- as evidenced by a main effect of Trial Type across the latter blocks of the Acquisition phase ($F(1,12) = 5.20$, $p = 0.04$). Figure 4 demonstrates the discrimination between the danger (AX) and safety (BX) cues at pre- and post-CPAP treatment timepoints.

Transfer of Inhibition Test (AB Test)

The ability to transfer inhibitory learning was measured during the Acquisition phase as in our previous studies (see Jovanovic et al., 2005). At pre-treatment, the participants did not exhibit significant discrimination between the AB test stimulus compound and the AX reinforced stimulus compound. Following CPAP treatment, participants displayed more robust discrimination between the CS+ and CS- ($F(1,10) = 6.86$, $p = 0.03$) and a trend toward better transfer of inhibitory learning (AX vs. AB: $F(1,10) = 4.86$, $p = 0.05$). Figure 4 shows fear-potentiated startle responses on the AB transfer of fear inhibition test at the pre- and post-treatment timepoints.

Extinction Learning Phase

At both the pre- and post-CPAP treatment timepoints, the extinction of fear-potentiated startle was assessed by presenting the previously reinforced AX stimulus compound without the airblast US. Prior to treatment, participants showed significant Extinction Learning as evidenced by a main effect of Block ($F(1,18) = 9.11$, $p = 0.007$). At post-treatment, participants also demonstrated within-session extinction learning as illustrated by a main effect of Block ($F(1,12) = 8.21$, $p = 0.01$).

The Extinction Learning phase consisted of six blocks with each block including 4 trials of each stimulus type. In the current study, the previously acquired fear-potentiated startle response was almost completely extinguished by the fourth block of Extinction at both the pre- ($F(1,18) = 7.25$, $p = 0.02$) and post-treatment ($F(1,12) = 10.2$, $p = 0.008$) timepoints (Figure 5).

These data exhibit an accelerated rate of extinction learning by this group regardless of the assessment time.

Extinction Recall Test

Twenty-four hours after the Extinction Training Session, participants underwent an Extinction Recall Test in which the previously reinforced AX+ stimulus compound was again presented without the airblast reinforcement. At both the pre- and post-CPAP treatment timepoints, there was a return of fear through spontaneous recovery (Pre: $F(1,18) = 5.23$, $p = 0.04$; Post: $F(1,10) = 5.46$, $p = 0.04$; Figure 5).

Patients diagnosed with co-morbid OSA and PTSD

We qualitatively examined the psychophysiological data collected from the subset of 8 patients that were diagnosed with OSA and PTSD. Statistical analyses were precluded by the underpowered low number of co-morbid OSA and PTSD patients identified in this sleep study. At the initial assessment, participants showed robust fear-potentiated startle to the reinforced conditioned stimulus (AX) as compared to noise alone (NA, or baseline; Figure 6). Additionally, the EMG startle responses suggest that this group of patients effectively discriminated between the reinforced (AX) and non-reinforced (BX) stimuli (Figure 7). Patients did not show significant inhibition of fear (AB) compared to the third block of Acquisition phase. Results from the Extinction Learning session demonstrated persistently elevated potentiated startle responses throughout the first 4 blocks. Decreased potentiated startle was noted at blocks five and six (Figure 8). During Recall, which occurred 24 hours after the Extinction Learning phase this subgroup of participants experienced a return of fear (Figure 8).

Discussion

Previous studies have suggested that co-morbidity of OSA and PTSD can lead to various health impairments (Lettieri et al., 2016). The data presented here represent the initial findings from an on-going pilot study investigating the effects of CPAP treatment on psychophysiological responses in veterans with OSA with and without PTSD. Results from the retrospective, exploratory study done by the Long Beach Sleep Clinic, as well as a recent publication by El-Solh and colleagues (2017), suggest that treating OSA via CPAP device can improve PTSD symptoms as measured by PCL scores. Our goal was to further investigate underlying neurophysiological biomarkers to better explain these improvements in symptomology. Based on previous work done by our group, we collected startle measurements using the conditional AX+/BX- Discrimination paradigm, as well as established Extinction and Extinction Recall paradigms (see Norrholm et al., 2008).

While the AX+/BX- Discrimination session and Fear Extinction sessions have been widely used as separate manipulations, this is the first study design that included both paradigms serially. We chose to use both procedures concurrently because there has phew studies investigating psychophysiological data collected in veterans with OSA. We aimed to capture a variation of physiological responses that have previously been studied in patients diagnosed with PTSD; it is common for this population to also be diagnosed with co-morbid OSA, with the result being an exacerbation of their symptoms.

During Acquisition phase, when the stimulus AX was paired with an aversive stimulus, subjects showed significant startle potentiation to the dangerous cue (AX+) during assessments

at both timepoints. These results support previous findings that a reduction in REM sleep prior to conditioning does not impair cued learning (Ruskin et al., 2004). Additionally, our study found that patients with OSA could successfully discriminate between danger (AX) and safety (BX) cues during the last two blocks of Acquisition phase. These results are suggestive of our participants' ability to learn the conditioning paradigm throughout the Acquisition session (Norrholm et al., 2015). Interestingly, we noted a greater ability to discriminate between the cues after CPAP treatment was completed. We propose that sleep can facilitate learning possibly due to participants' enhanced attention to the task (Walker and Stickgold 2004). Overall, the lack of a reduction in startle to the AX+ stimulus at the post-treatment timepoint argues against habituation to the paradigm or practice effects. Furthermore, our research group counterbalanced both the shapes and the colors presented to our subjects at the second assessment; hence, improvements in discrimination between danger and safety cues should occur largely independent of practice effects.

The current study demonstrated that CPAP treatment promoted inhibitory fear learning as our results were approaching statistical significance ($p=0.05$) in the Transfer of Inhibition Test (AB Test) post-treatment. These results were most likely due to the large variation in our participants' startle responses during the final block of the Acquisition phase, suggesting the importance of continuing to recruit participants with the objective of a larger scale, well-powered investigation. Nevertheless, the trend towards increased inhibition of fear in our results support previous findings, as the presentation of the conditioned excitor, stimulus A, together with the conditioned inhibitor, stimulus B, greatly reduced the conditioned startle response compared to the conditioned response to AX (Myers and Davis 2002; Myers and Davis

2004). CPAP treatment improves sleep quality and increases the duration of REM sleep. Some literature suggests that sleep deprivation leads to greater amygdala activation, while it reduces amygdala-medial prefrontal (mPFC) connections in response to emotional stimuli (Pace Schott et al., 2009). Perhaps, the lack of fear inhibition prior to CPAP treatment could be attributed to interferences with the mPFC-amygdala circuitry as a result of interrupted sleep in patients with obstructive sleep apnea.

Results from the fear Extinction phase demonstrate almost complete extinction by the third block. Norrholm and colleagues (2008) previously demonstrated that healthy participants do not begin to significantly decrease startle responses to the previously reinforced conditioned stimulus until the second half of the Extinction Learning phase (extinction blocks 4, 5 and 6). Based on this study, as well as other previously published work from our group, we expected the startle responses to be fully extinguished at approximately block 5 and 6 (final two blocks) of the paradigm. However, our subjects were markedly different as they demonstrated an accelerated rate of Extinction Learning and showed relatively low startle magnitudes by block 3 of the session at both pre- and post-treatment timepoints.

It is important to note that within our protocol, the Extinction Learning phase occurred after the Transfer of Inhibition Test, which was part of the AX+/BX- Discrimination paradigm. We propose that the Transfer of Inhibition test might have “primed” the inhibitory responses observed throughout extinction, and as such, facilitated a decrease in startle responses. Research examining the role of the prefrontal cortex on amygdala-driven responses is quite controversial. Some report that lesions to the mPFC result in impaired extinction (Morgan et al.,

1993), while others found no differences between lesioned and control animals (Quirk et al., 2000). Nevertheless, this brain region appears to play a role in the inhibition of previously conditioned fear responses. In relation to our results, we postulate that completing the Transfer of Inhibition test prior to the Extinction Learning session enhanced the activation of the mPFC, which acts through inhibitory projections onto the amygdala (Quirk et al., 2006). This increase in GABA-ergic inputs to the amygdala could explain our observed decrease in the expression of conditioned fear during extinction.

Our set of participants demonstrated a spontaneous recovery of fear during the Extinction Recall test both pre- and post-CPAP treatment. Nevertheless, these results do not necessarily suggest an impaired ability to recall Extinction Learning. Healthy participants are likely to experience a return of conditioned fear when there is change of context (renewal), after a period of time (spontaneous recovery) and if they undergo sudden re-exposure to the aversive stimulus (reinstatement; Warren et al., 2013). Warren and colleagues (2013) demonstrated that healthy participants show a return of fear known as a spontaneous recovery after the reduction in the magnitude of conditioned responses throughout Extinction, which is similar to our results. This is because both the acquisition of conditioned fear and the extinction of fear are context-dependent. The context in our study remained the same for all phases of the experiment.

We further investigated a small sample of patients who were diagnosed with OSA and co-morbid PTSD at their initial assessment in the clinic. The Acquisition data was qualitatively similar to the full analysis completed in patients that were diagnosed with OSA and underwent

CPAP treatment. However, Extinction Learning appears to be impaired in a manner similar to what our group has observed previously in combat veterans with PTSD (Norrholm and Jovanovic, 2011). This subgroup of patients required a longer period of time to extinguish fear-potentiated startle unlike the rapid extinction that we now reported in the current study in patients with OSA. These results could be explained by previous reports showing increased activity in the amygdala during Extinction as well as impaired inhibition of the amygdala by the prefrontal cortex in patients with a history of trauma or PTSD (Norrholm et al., 2011).

Our results also showed that this subgroup of patients displayed a return of fear 24 hours after Extinction. These results could be explained by Milard and colleagues (2009) who noted that patients with PTSD have impaired Recall of Extinction memory accompanied by a decrease in hippocampus and ventromedial prefrontal cortex (vmPFC) activation but an increase in dorsal anterior cingulate cortex (dACC) activation compared to healthy controls. Activation of the vmPFC is involved in Extinction Learning and Recall of Extinction memories; hence the decrease in this structure's activity in patients with PTSD suggests a decrease in top-down inhibition of the amygdala resulting in greater conditioned fear responses (Pace Schott et al., 2015). However, it is important to note that the sample size of subjects with OSA and PTSD was very small and had a lot of variation in startle responses. Additionally, as noted earlier, spontaneous recovery can occur in healthy participants, so these results cannot solely be attributed to the PTSD diagnosis. Statistical analysis was not performed in this group because our study was underpowered. Finally, when examining the Extinction data for this subgroup at the post-treatment time point, we did notice that the participants had a decrease in startle to the previously reinforced conditioned stimulus; however, because of the extremely small

sample size this data is only speculative at this point. While studies have shown improvements in PTSD severity after the use of CPAP, some have also addressed the major issues of adherence in this population. Lettieri and colleagues (2016) reported that patients with OSA and co-morbid PTSD had significantly lower PAP adherence. In our study the average time of CPAP treatment was approximately 8 weeks. While some patient's had improvements in sleep after 4 weeks of treatment others improved after week 12. These differences were possibly due to the variability in the participant's adherence to the treatment.

Subjects that were diagnosed with OSA but did not undergo CPAP treatment were few in number and showed highly variable startle responses, which again prevented us from performing meaningful statistical analyses. As mentioned earlier, this is an ongoing larger study and the clinic continues to treat many veteran patients diagnosed with OSA with and without co-morbid PTSD.

Future Directions and Limitations

In recent years, there has been initiative within the Veterans Affairs Healthcare System to better screen for OSA and PTSD (Colvonen et al., 2015; Forbus and Kelly, 2015). As a result more patients are screened for these disorders, allowing studies like ours to further explore the underlying neurobiology leading to general medical and psychiatric impairments. While the goal is to better understand veteran health and disease and to improve treatment approaches, this can affect clinical research study design. For example, the original goal for clinicians at the Sleep Clinic at Long Beach VA was to treat patients that presented with symptoms of OSA. A psychiatrically healthy control group was not included in the initial study design and its need

became apparent as the number of OSA and PTSD-affected veterans increased. At this point, the number of control participants available for study was limited and it was not statistically appropriate to attempt quantitative data analyses. The data that are presented herein represent the initial results from our ongoing study and we focused on the group of patients that were diagnosed with OSA and successfully treated using CPAP therapy. Our results show interesting trends, as described above, however there is a need to increase the number of participants and do further research in this group of patients to better understand the effects we have revealed. A well-powered study with at least 20 patients per group is a long-term goal of this project.

Based on a review written by Pace-Schott and colleagues (2015), it appears that most sleep studies have focused on skin conductance response (SCR) measures as psychophysiological indices of fear conditioning, Extinction Learning, and Extinction Recall paradigms. It would be of great interest to concurrently measure SCR data and EMG (fear-potentiated startle) responses. While SCR measures overall autonomic nervous system-driven arousal to various stimuli, EMG responses are valence specific and allow for the discrimination between aversive and pleasant stimuli. Hence, it would be important to analyze both measures in future sleep studies. We also collected expectancy measures in our participants that were measured using a button box keypad. On the keypad, participants indicated whether or not they expected the US to follow a CS by pressing a specific button for positive expectancy, negative expectancy, or uncertainty. The inclusion of SCR and expectancy measures in future studies will provide greater insight into the cognitive and physiological effects of CPAP treatment on fear learning.

While categorical PTSD diagnosis was determined by clinicians using DSM-5 criteria, it would be interesting to further explore our patients' individual clinical presentations. As mentioned earlier, PTSD is a heterogeneous disorder and presents in different symptom clusters. While some patients may demonstrate hyperarousal and an increase in intrusive memories, others may primarily present clinically with avoidant behaviors. Based on our body of work, we would expect patients with symptoms of hyperarousal to exhibit worse outcomes in the Extinction Learning tasks similar to what was reported in prior sections of this work. Future studies will examine fear learning and treatment-related changes by examining heterogeneous PTSD at the level of each symptom cluster type.

Conclusion

Veterans with co-morbid OSA and PTSD can show manifestations of poor sleep quality as well as a worse general medical health conditions and increased PTSD symptom severity. Our results demonstrate the possible benefits to treating OSA with a CPAP therapy as it may improve the patients' ability to discriminate between danger and safety cues. Additionally, we propose that enhancing prefrontal cortex activity prior to Extinction Learning may help facilitate the inhibition of fear. Further research is necessary to further investigate and replicate the results of our study with the long-term hope of improving current protocols for treatment in patients with these disorders.

Figures and Tables

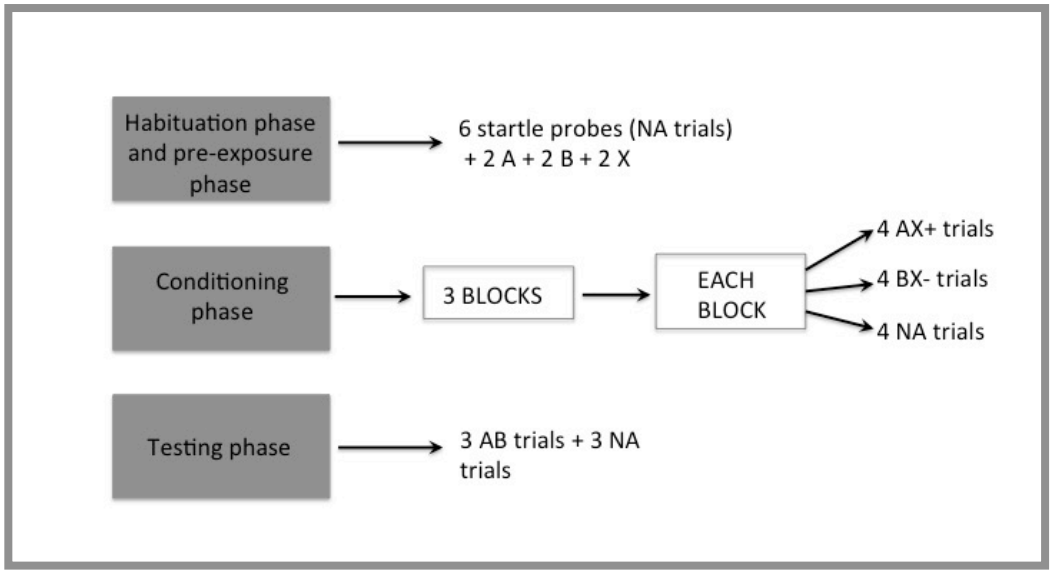

Figure 1. Diagram of the AX+/BX- startle session used in our paradigm (adapted from Jovanovic et al., 2005). AX+ is the reinforced stimulus, BX- is the non-reinforced stimulus. Noise alone, NA.

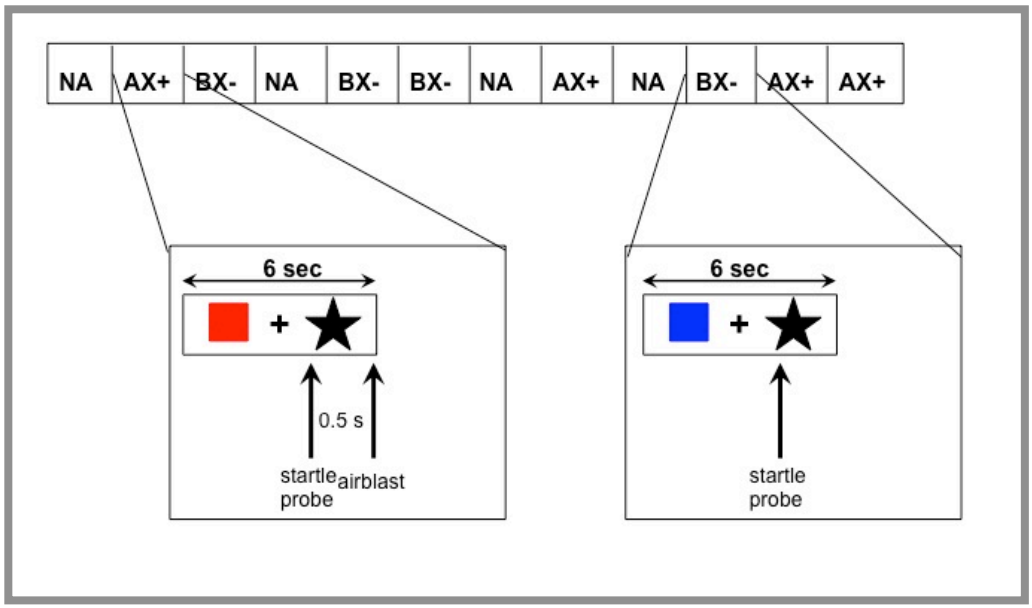

Figure 2. Diagram of the Trials During AX+/BX- Session. AX+ and BX- trials were placed randomly within each block. AB trials were structured similarly to BX- (adapted from Jovanovic et al., 2005). Shapes and colors were alternated following treatment. The conditioned stimuli were counterbalanced across participants.

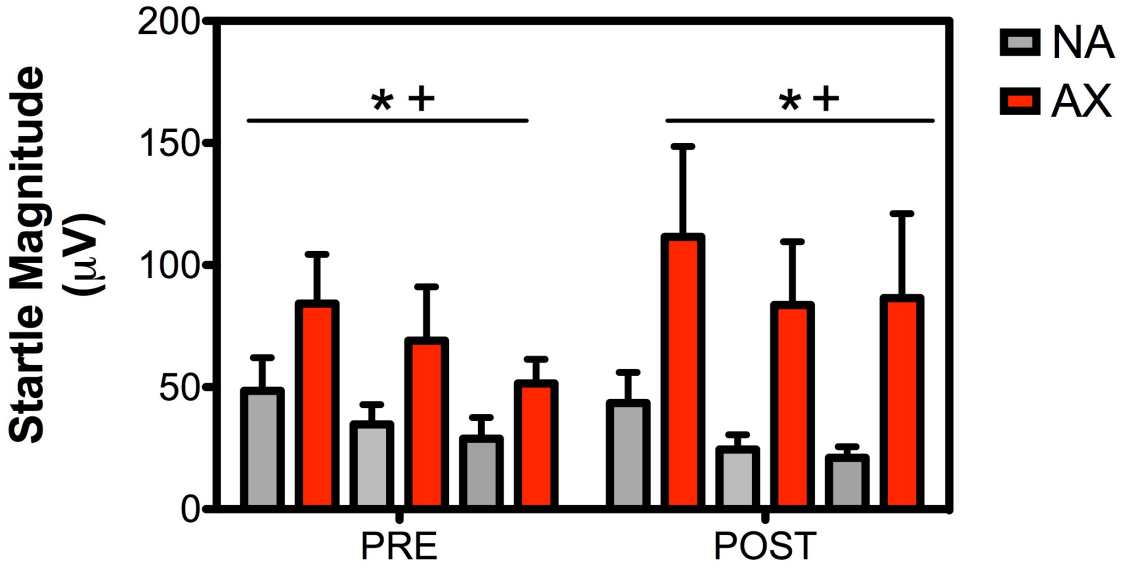

Figure 3: Fear Acquisition: Fear-Potentiated Startle. Mean startle magnitude to noise alone (NA) and in the presence of the aversive stimulus (AX+) pre- and post-CPAP treatment in patients with OSA. * Indicates main effect of Block ($P<0.05$). + indicates main effect of Trial ($P<0.05$).

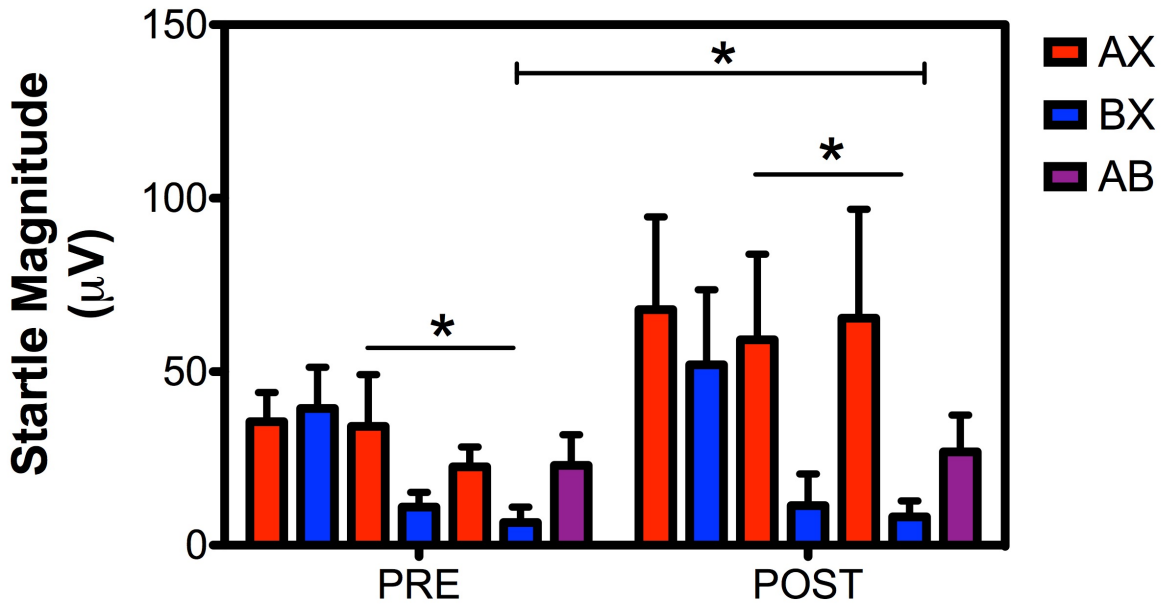

Figure 4: Fear Acquisition: CS Discrimination and Transfer of Inhibition Test. Startle magnitude is defined by the Difference Score [Mean startle response to CS (AX, BX, AB)] – [Mean startle response to noise alone (NA)]. Significant effect of Trial ($P<0.05$) across second and third blocks pre- and post-CPAP treatment. Improvement in discrimination between AX+ and BX- in third block at post-treatment timepoint compared to pre-treatment timepoint. Approaching significant inhibition of fear (AB) post-treatment ($P=0.05$).

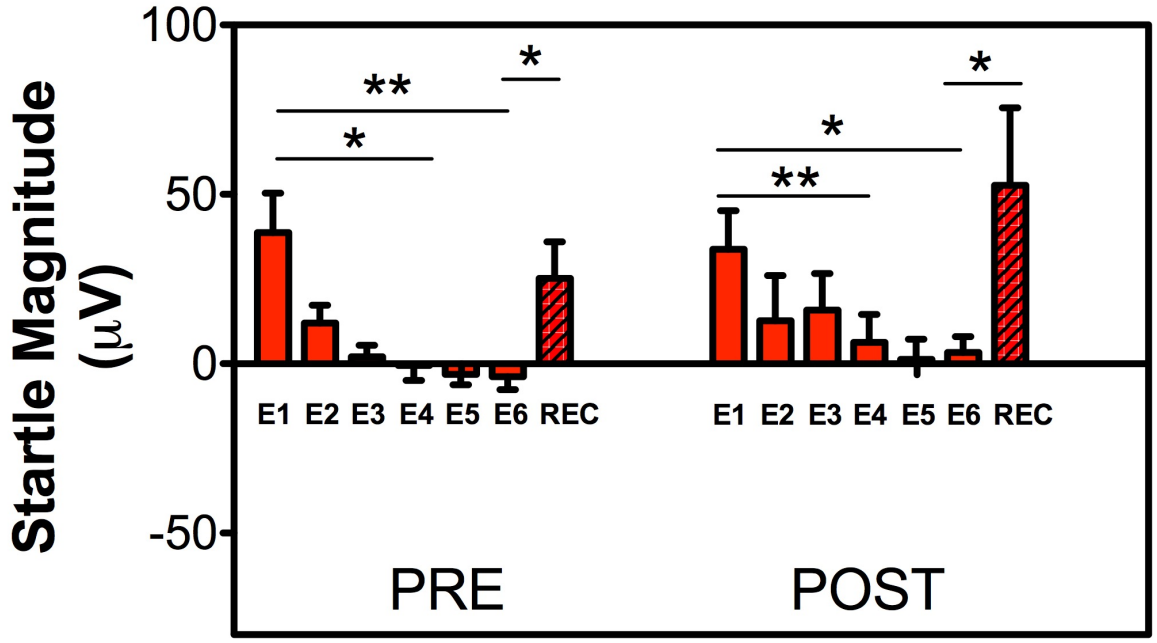

Figure 5: Extinction Learning and Recall Test. Main effect of Block demonstrated during Extinction Learning pre- and post-CPAP treatment. During Early and Mid – Extinction individuals displayed significant reductions in startle magnitude shown by main effect of Block pre- and post-treatment (* P <0.05, ** P< 0.01). Subject had a significant increase in startle magnitude twenty-four hours after the last block of Extinction Learning during Extinction Recall Test (P<0.05).

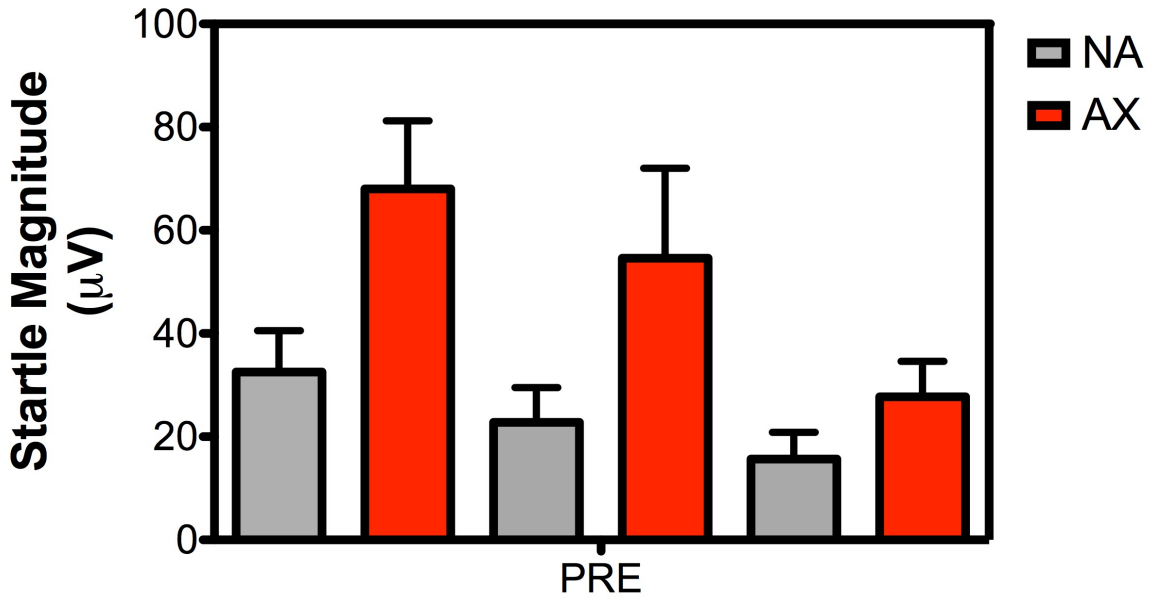

Figure 6: Fear Acquisition (OSA and PTSD): Fear-Potentiated Startle. Mean startle magnitude to noise alone (NA) and in the presence of the aversive stimulus (AX+) pre-CPAP treatment. Patients with OSA and PTSD demonstrate response potentiation to AX. Over time the patients show a trend towards habituation.

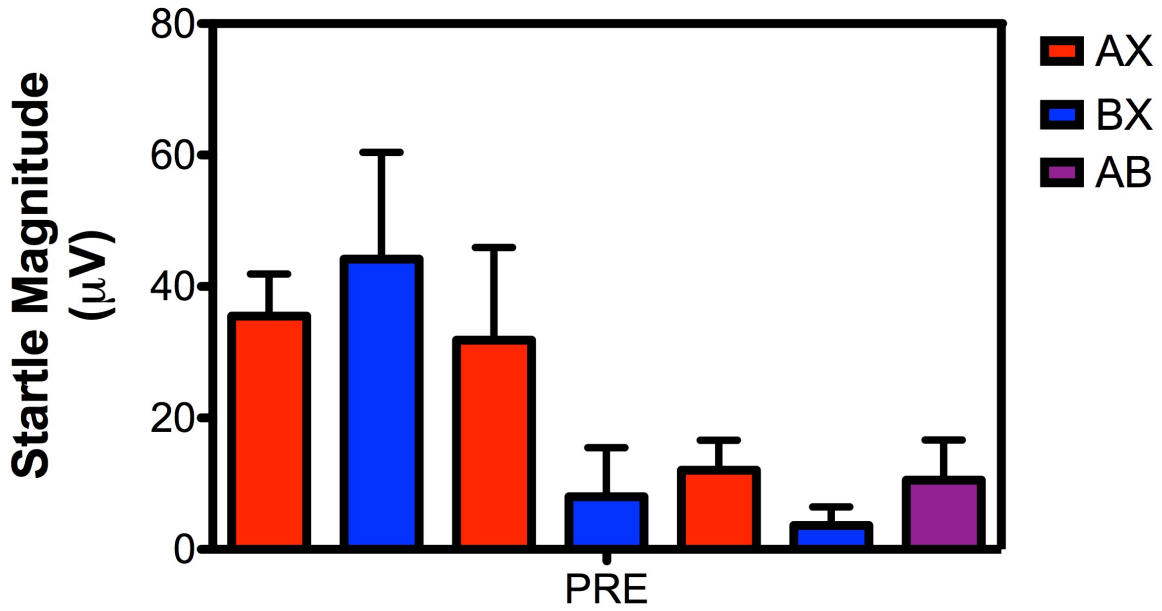

Figure 7: Fear Acquisition Test (OSA and PTSD): CS Discrimination and Transfer of Inhibition. Startle magnitude is defined by the Difference Score [Mean startle response to CS (AX, BX, AB)] – [Mean startle response to noise alone (NA)]. Subjects diagnosed with OSA and PTSD discriminated between AX and BX. Subjects displayed no evidence of inhibition of fear responses (AB).

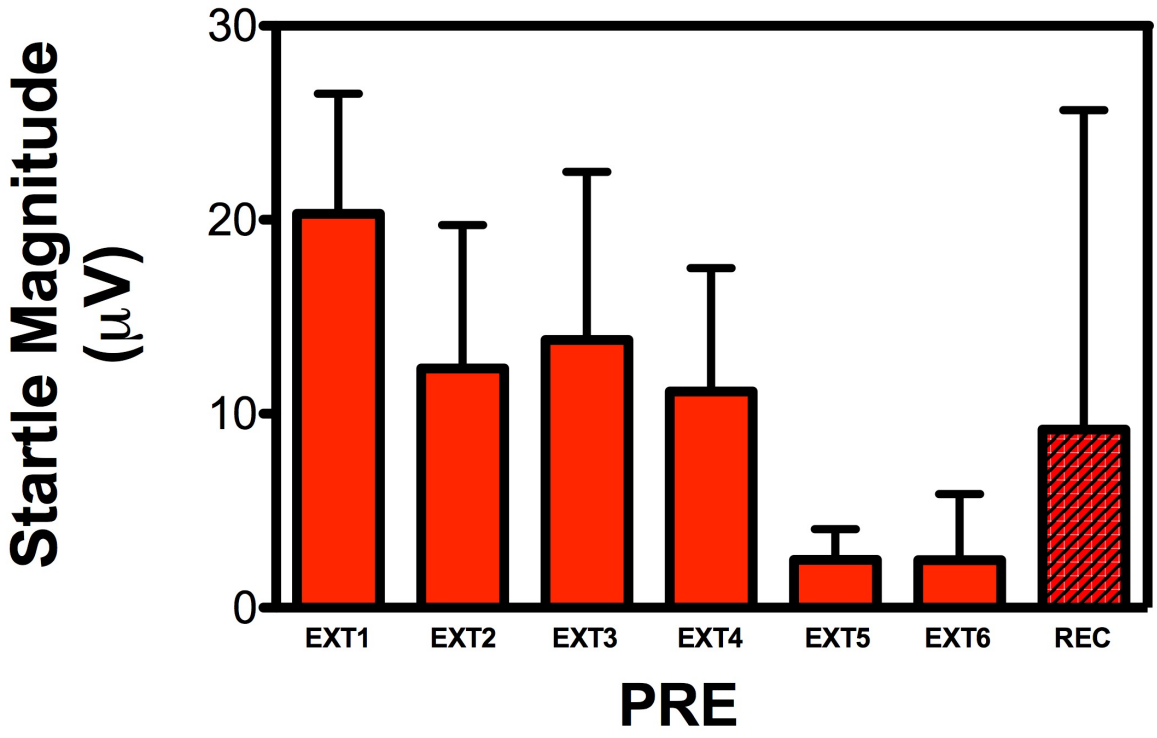

Figure 8: Extinction Learning and Extinction Recall Test (OSA and PTSD). Subjects diagnosed with OSA and PTSD demonstrated impaired fear extinction. Twenty-four hours after Extinction Learning patients display return of fear during Extinction Recall Test.

Effects of CPAP treatment (n=12)		
	Pre	Post
PCL	65 ± 9	37 ± 8
BDI	25 ± 8	15 ± 13

Table 1. Retrospective Chart Review of Patients with OSA. There was a reduction in PTSD severity as measured by PCL by 43%.

Patient Demographic and Clinical Variables (n=41)	
Gender	Female: 4
	Male: 37
Age, years	24-56
Ethnicity	White: 12
	Hispanic: 14
	Black: 9
	Asian, Pacific Islander: 4
	Native American: 2
OSA diagnosis	32
OSA and PTSD diagnosis	8
Sleep Study Measures (all participants)	
Lowest Oxygen Saturation during day sleep, %	81
Apnea-Hypopnea Index, events/h	48
Sleep Efficiency, %	85

Table 2. Demographic and Clinical information. Information gathered from all participants. Out of 41 subjects 8 participants were healthy, 9 were diagnosed with OSA and did not receive treatment, 23 had OSA and were treated with a CPAP device and 1 was excluded prior to data collection.

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